Elov4 5-bp deletion knock-in mouse model for Stargardt-like macular degeneration demonstrates accumulation of ELOVL4 and lipofuscin

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1. Introduction

Stargardt-like macular degeneration (STGD3) is inherited in an autosomal dominant manner. Patients affected with STGD3 exhibit progressive central vision loss with onset in the second decade of life. Fundus examination of these patients reveals retinal pigment progressive central vision loss with onset in the second decade of life. Fundus examination of these patients reveals retinal pigment degeneration demonstrating accumulation of ELOVL4 and lipofuscin.

The mechanism underlying photoreceptor degeneration in autosomal dominant Stargardt-like macular degeneration (STGD3) due to mutations in the elongation of very long chain fatty acids-4 (ELOVL4) gene is not fully understood. To evaluate the pathological events associated with STGD3, we used a mouse model that mimics the human STGD3 phenotype and studied the progression of retinal degeneration. Morphological changes in the retina of Elov4 5-bp deletion knock-in mice (E_mut/C0) were evaluated at 22 months of age. The localization of ELOVL4, and the expression pattern of inner retinal tissue marker proteins, and ubiquitin were determined by immunofluorescence labeling of retinal sections. Levels of the retinal pigment epithelium (RPE) lipofuscin fluorophores were measured by quantitative HPLC. Morphological evaluation of the retina revealed an accumulation of RPE debris in the subretinal space. A significant increase in the amount of ELOVL4 was observed in the outer plexiform layer in E_mut/C0 mice compared to controls. Apart from the accumulation of ELOVL4, E_mut/C0 mice also exhibited high expression of ubiquitin in the retina. Analysis of lipofuscin fluorophores in the RPE showed a significant elevation of A2E and compounds of the all-trans-retinal dimer series in retinas from four and ten month old E_mut/C0 mice compared to wild-type littermates. These observations suggest that abnormal accumulation of ELOVL4 protein and lipofuscin may lead to photoreceptor degeneration in E_mut/C0 mice.

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age (Vasireddy et al., 2006). Mice carrying the 5-bp deletion mutation in the homozygous state die due to a defective skin permeability barrier. Studies on these mice revealed involvement of ELOVL4 in the synthesis of fatty acids with chain length longer than C > 26 (Vasireddy et al., 2007). To gain insight into the role of ELOVL4 in the pathophysiology of STGD3 and progression of retinal degeneration, we further characterized E_mut+/− mice, and evaluated the changes of retinal pigment epithelium (RPE) and inner retinal layers of E_mut+/− mice between 4 and 22 months of age. Electoretinographic response of these mice was measured at age 22 months. Quantification of the RPE lipofuscin fluorophore, A2E and lipofuscin pigments of the all-trans-retinal dimer series in eye cups of E_mut+/− mice revealed a significant elevation of these fluorophores in E_mut+/− retina compared to controls. Immunolabeling of E_mut+/− mice retinal sections detected an accumulation of ELOVL4 in the dendritic tips of rod bipolar cells. No changes were observed in the ERG response and architecture of inner retinal layers at older age. Together these observations suggest that accumulation of ELOVL4 and lipofuscin in the retina may lead to the progression of photoreceptor degeneration in mice carrying the Elov4 5-bp deletion in the heterozygous state.

2. Materials and methods

Anti-ELOVL4 antibodies (1:500 dilution, Abcam, Cambridge, MA), The affinity-purified ELOVL4 antibody was described previously (1:500 dilution (Lagali et al., 2003), anti- PKC-alpha antibodies (Sigma–Aldrich, St. Louis, MO), anti-PSD-95 antibody, Alexa fluor-448 (1:1000 dilution, Invitrogen-Molecular probes, Carlsbad, CA); Alexa fluor-555 (1:2500 dilution, Invitrogen-Molecular probes, Carlsbad, CA), Protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO), anti-ubiquitin antibody (1:500 dilution, Sigma–Aldrich, St. Louis, MO); paraformaldehyde (EMS), normal goat serum (Vector labs, Burlingame, CA).

2.1. Animal maintenance and tissue preparation for immunohistochemistry

Animals were maintained according to the ARVO policy for animal maintenance and following the guidelines of institutional Animal Care and Use Committee. Breeding and genotyping of Elov4 knock-in animals was described elsewhere (Vasireddy et al., 2006). All animals were maintained in 12 h light and 12 h dark cycles. To genotype for murine Rpe65 Leu450Met variant, DNA derived from mouse tails was PCR-amplified with forward 5'-ACCAGAAATT TGGAGGAAAC-3' and reverse 5'-CCCTTACA TTGACAAGT CTCA-3' primers. Digestion of the resulting 545-bp product with 50-fold excess of MwoI restriction enzyme (New England Biolabs, Ipswich, MA) yielded fragments of 180 and 365 bp in the presence of the sequence variant corresponding to Leu450. Undigested 545-bp product was resolved with the Met450 variant and 180, 365 and 545-bp products were indicative of heterozygosity. To avoid variation in lipofuscin content in E_mut+/− mice due to Rpe65 Leu450 Met variation, all mice used for lipofuscin estimation were genotyped for this change.

2.2. Histology

Twenty two month old E_mut+/− mice and age matched littermate control animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. After enucleation, the eyes were immersed in the same fixative for 24 h. After fixation, the eyes were thoroughly washed with PBS and embedded in OCT and processed for sectioning.

2.3. Immunofluorescence labeling of retinal sections

Immunofluorescence labeling of retinal sections was performed as described earlier (Vasireddy et al., 2006). Briefly, 10 μm thick cryosections were cut from paraformaldehyde (4% w/v)-fixed and OCT-embedded eye cups. The sections were treated with proteinase K, and non-specific binding of the antibody was blocked using 10% normal goat serum in PBS containing 0.1% Triton-X 100 for 1 h at room temperature (RT), and then incubated overnight at 4 °C with diluted primary antibody mixture. Sections were then washed and incubated in appropriate secondary antibody mixture for 1 h at RT. Following thorough washes with PBS containing Triton-X 100 and PBS alone, slides were mounted in mounting media containing DAPI and observed with a Zeiss confocal microscope.

2.4. Analysis of lipofuscin pigments

Posterior murine eye cups including sclera, choroid, RPE, and neural retina, were homogenized using a glass tissue grinder; were extracted three times with chloroform/methanol (2:1) and then passed through a reverse phase cartridge (C18 Sep-Pak, Millipore) with 0.1% TFA in methanol. The extract was dried under argon, redissolved in methanol, and analyzed by reverse-phase HPLC using an Alliance System (Waters, Corp, Milford, MA) equipped with 2695 Separation Module, 2996 Photodiode Array Detector, a 2475 Multi ëFluorescence Detector. Chromatographic separation was achieved on analytical scale Atlantis® dC18 (3 μm, 4.6 × 150 mm, Waters, USA) and Delta Pak® C4 (5 μm, 3.9 × 150 mm, Waters, USA) columns and an acetonitrile and water gradient with 0.1% trifluoroacetic acid (gradient for dC18: 90–100%, 0–10 min; 100% acetonitrile, 10–20 min; gradient for C4: 75%, 0–5 min; 75–100%, 5–20 min; flow rate, 0.8 ml/ min; monitoring at 430 and 510 nm; 10 μl injection volume). Integrated peak areas were determined using Empower® software, and picomolar concentrations were calculated by reference to external standards of synthesized compounds, the structures of which have been corroborated (Parish et al., 1998; Fishkin et al., 2005; Kim et al., 2007b). For quantitation, A2E, isoA2E and unconjugated all-trans-retinal dimer were monitored at 430 nm and all-trans-retinal dimerohexadecyl ethanolamine and all-trans-retinal ethanolamine were monitored at 500 nm.

3. Results

3.1. Morphology of E_mut+/− mice retina

Earlier we reported abnormalities in the RPE and photoreceptor layer of the retina of E_mut+/− mice at age 2–15 months. To further evaluate the progression of retinal degeneration in these mice, we investigated the histological changes in the retinas of 22 month old E_mut+/− and control mice. Morphological evaluation demonstrated abnormalities in the RPE and photoreceptors. An accumulation of RPE debris in the subretinal space was observed, consistent with our observations in E_mut+/− mice at younger ages. Specifically, pigment granules of RPE origin were often found among photoreceptor outer segments. Moreover inner segments were swollen in the E_mut+/− mice compared to wild-type littermate controls (Fig. 1). Although these changes were consistent, variation was noted in the amount of RPE debris between animals.

3.2. Lipofuscin accumulated in the RPE of E_mut+/− mice

Ultrastructural analysis of the RPE of 8 month old E_mut+/− mice demonstrated an increased number of electron dense inclusion bodies compared to age matched controls (Fig. 2). The structure of these electron dense inclusion bodies resembled lipofuscin
granules. The number of these lipofuscin granules is significantly higher in E_mut+/− mice compared to controls (Fig. 3). Other striking features that are present in the RPE of E_mut+/− mice are the presence of many cytoplasmic vacuoles, enlarged melanosomes and the lack of structural definition of the basal infoldings of the RPE (Fig. 2).

### 3.3. Accumulation of lipofuscin pigment in the retina of E_mut+/− mice

To further characterize the accumulated lipofuscin in the Elovl4 5-bp deletion mutant, we analyzed extracts of posterior eye cups harvested from E_mut+/− and wild-type mice for their content of lipofuscin granules. The number of these lipofuscin granules is significantly higher in E_mut+/− mice compared to controls (Fig. 3). Other striking features that are present in the RPE of E_mut+/− mice are the presence of many cytoplasmic vacuoles, enlarged melanosomes and the lack of structural definition of the basal infoldings of the RPE (Fig. 2).
All mice were Rpe65 Leu450Met variant: * not genotyped for Rpe65 trans heterozygous; ** not genotyped for Rpe65 variant B. Levels of A2E and isoA2E (summed) together with total (summed) all-trans retinal –ethanolamine[all-trans-retinal dimer-phosphatidylethanolamine and all-transretinal –ethanolamine] in wild-type versus E_mut/C0 mice, compared to control mice retina with anti-ELOVL4 antibodies showed co-localization of ELOVL4 and PKC in the dendritic tips of rod bipolar cells.

Similarly ELOVL4 showed co-localization with PSD-95. PSD-95 (Fig. 7) antibodies along with anti-ELOVL4 antibodies showed co-localization of ELOVL4 and PKC in the dendritic tips of rod bipolar cells.

To examine the localization of ELOVL4 in E_mut/C0 mice, we performed immunohistochemical labeling of retina from 4 month old E_mut/C0 mice and age matched controls. Consistent with our earlier observation, intense labeling of ELOVL4 was detected in the OPL region of the retina in E_mut/C0 mice as compared to controls, suggesting the accumulation of ELOVL4 protein in these animals (Fig. 5).

To further confirm the localization of ELOVL4 in OPL, retinal sections were co-labeled with rod bipolar cell specific marker, protein kinase C- alpha (PKC) (Fig. 6) and PSD-95(Fig. 7) antibodies along with anti-ELOVL4 antibodies. Co-immuno labeling of retinal sections of E_mut/C0 mice and control mice retina with anti-ELOVL4 and anti-PKC antibody showed co-localization of ELOVL4 and PKC in the dendritic tips of rod bipolar cells.

3.4. ELOVL4 accumulates in the retina

To examine the localization of ELOVL4 in E_mut/C0 mice, we performed immunohistochemical labeling of retina from 4 month old E_mut/C0 mice and age matched controls. Consistent with our earlier observation, intense labeling of ELOVL4 was detected in the OPL region of the retina in E_mut/C0 mice as compared to controls, suggesting the accumulation of ELOVL4 protein in these animals (Fig. 5).

Fig. 4. Quantitation of the lipofuscin pigments in E_mut/C0 mouse: A. A2E and isoA2E in posterior eye cups were measured by quantitative HPLC with monitoring at 430 nm. Levels of A2E and isoA2E were summed. Values (mean ± SEM) are based on 2-5 replicates per age and each sample was obtained by pooling 4 eyes. Genotyping for the Rpe65 Leu450Met variant: * Rpe65 450Leu (homozygous) and 450LeuMet (heterozygous) were pooled in equal numbers in wild-type and E_mut/C0 (homozygous); ** Rpe65 450Leu (homozygous); *** not genotyped for Rpe65 variant B. Levels of A2E and isoA2E (summed) together with total (summed) all-trans-retinal dimer series compounds [alltrans-retinal dimer, all-trans-retinal dimer-phosphatidylethanolamine and all-transretinal –ethanolamine] in wild-type versus E_mut/C0 mice and in Abca4+/– mice. All mice were Rpe65Leu450 and 4 months of age. Values (mean ± SEM) are based on 2–3 replicates per age and each sample was obtained by pooling 4 eyes. Pigments were identified by co-injection with authentic standard. P values determined by unpaired t-test.

Fig. 5. ELOVL4 protein accumulates in the retina of E_mut/C0 mice: Immunofluorescence analysis of retinal sections from 4 month old controls (A) and E_mut/C0 mice (B) demonstrated an accumulation of ELOVL4 protein (red) in the OPL of E_mut/C0 mice retina. Nuclei are labeled with DAPI. ONL: Outer nuclear layer, OPL: Outer plexiform layer, INL: Inner Nuclear layer. Scale bar – 10 µm.
3.5. Expression of ubiquitin in the retina

In cells, aberrant and/or misfolded proteins are degraded and removed by the ubiquitin proteasomal system. If the cellular burden to remove the aberrant protein increases, unprocessed protein will accumulate and lead to cellular stress. Enhanced expression of ubiquitin and ubiquitination of proteins can serve as an indicator of saturated ubiquitin proteasomal degradation (Alves-Rodrigues et al., 1998; Bence et al., 2001; Saliba et al., 2002). In order to compare the levels of ubiquitin in $E_{mut}^{-/-}/C0$ and control retinas, we evaluated the expression levels of ubiquitin in $E_{mut}^{-/-}/C0$ mice by immunohistochemistry (Fig. 8). Ubiquitin immunoreactivity was observed all through the inner retina in both $E_{mut}^{-/-}/C0$ mice and the control mice. However, the intensity of staining was higher in the IS of the photoreceptors in $E_{mut}^{-/-}/C0$ mice than in age matched controls.

4. Discussion

The progressive accumulation of high amounts of fluorescent lipofuscin compounds in RPE has been observed in inherited retinal disorders (Eagle, 1984). The bis-retinoid pyridinium compound A2E and its photoisomer isoA2E are major components of lipofuscin and these compounds have been structurally characterized (Parish et al., 1998). A2E forms in photoreceptor outer segments via a multi-step biosynthetic pathway that involves reactions of all-trans-retinal and phosphatidylethanolamine and the formation of an intermediate, dihydroA2PE (A2PE-H2) (Parish et al., 1998; Liu et al., 2000; Ben-Shabat et al., 2002; Kim et al., 2007a). Phosphate cleavage of A2PE within RPE lysosomes releases A2E. Enhanced accumulation of A2E and the isomers of A2E were reported in the patients of Stargardt and Stargardt-like macular degeneration. However, the amount of lipofuscin accumulation was observed to be less in Stargardt-like macular degeneration patients compared to Stargardt macular degeneration patients. The accumulation of A2E and isoA2E in RPE cells is increased in $Abca4^{-/-}/C0$ null mutant mice (Mata et al., 2000; Kim et al., 2007b) and considerably more moderate increases in A2E and isoA2E were also observed here in $E_{mut}^{-/-}/C0$ mice. An additional family of lipofuscin compounds has been characterized, the all-trans-retinal dimer series, that includes all-trans-retinal dimer-PE, all-transretinal dimer-E and unconjugated all-trans-retinal dimer (Fishkin et al., 2005; Kim et al., 2007b). The lipofuscin pigment all-trans-retinal dimer-PE is the most prominent member of this series and is notable not only because it is increased in $Abca4^{-/-}/C0$ mice, but also because in this mouse model of recessive Stargardt disease, all-trans-retinal dimer-PE is more abundant than A2E. While total all-trans-retinal dimer pigment was increased in $E_{mut}^{-/-}/C0$ mice relative to wild-type, these pigments were considerably less abundant than in $Abca4^{-/-}/C0$ mice. This difference is potentially significant. The mechanism by which ABCR (ABCA4) mutations predispose to higher lipofuscin levels in autosomal recessive Stargardt disease is understood, the reduction in ABCR protein activity making all-trans-retinal more readily available for formation of the bis-retinoid compounds that constitute lipofuscin.

![Fig. 6. Accumulation of ELOVL4 protein in synaptic terminals of bipolar cells: Cross sections of retina from 4 month old $E_{mut}^{-/-}/C0$ mice (A) and Wt control (B) double stained with the bipolar cell marker PKC-alpha antibody (green) and ELOVL4 antibody (red). ELOVL4 was observed to be co-localized with PKC-alpha antibody at the dendritic tips of rod bipolar cells. Panel C is a higher magnification image showing the localization of ELOVL4 in rod bipolar cells. Scale is 50 μM.](image-url)
pigments. Conversely, the relationship between mutations in ELOVL4 and RPE lipofuscin formation is not known.

Although ELOVL4 is observed to be localized in both rod and cone photoreceptors, E_mut+/− mice demonstrated progressive loss of cones and STGD3 patients showed central vision loss consistent with cone abnormalities. Accumulation of ELOVL4 protein that was observed in OPL regions is significantly higher in dendritic tips of rod bipolar cells. The relationship between accumulation of ELOVL4 protein in specific regions of OPL and cone degeneration is not understood. It has been demonstrated in various animal models that

Fig. 7. Accumulation of ELOVL4 in the OPL of E_mut+/− mice: Immunofluorescence images of 4 month old control (A,C,E) and E_mut+/− mice (B,D,F) retinal sections labeled with anti-ELOVL4 antibody (red) and PSD-95 (green). Immunofluorescence of ELOVL4 labeling (red) is co-localized with the PSD-95 antibody (green) labeling in the outer plexiform layer region in E_mut+/− mice compared to age matched controls. Scale is 50 μM.
abnormal accumulation of protein in OPL leads to the degeneration of inner retina and affects the structure and function of second order neurons. The dysfunction in the retina in many of these animal models is evidenced by the altered morphology of the inner retina and reduction in electroretinographic (ERG) b-wave amplitudes. In order to evaluate the alterations in synapses, ERG was recorded in \( E_{mut}^{+/+} \) mice and compared with age matched controls. The ERG response of \( E_{mut}^{+/+} \) mice did not indicate a significant physiological alteration up to age 24 months (data not shown).

Further evaluation of the inner retina with antibodies specific to outer plexiform layer, amacrine cells, horizontal cells, inner plexiform layer, and ganglion cell marker proteins did not reveal significant abnormalities in the structure and organization of inner retina in \( E_{mut}^{+/+} \) mice (data not shown). These observations suggest that accumulation of ELOVL4 protein in IS and OPL may not cause impairment of inner retinal function. These findings are consistent with the retinal phenotype observed in patients with Stargardt-like macular degeneration.

Abnormal protein accumulation has been reported in different forms of macular degenerations, retinal degenerations and other neurodegenerations (Rajan et al., 2001; Saliba et al., 2002; Soto, 2003; Klenotic et al., 2004; Soto and Estrada, 2008). However the molecular pathology underlying these diseases due to protein accumulation is not clear. Altered protein trafficking and accumulation was observed in retinal tissue due to mutations in the EFEMP1, Rhodopsin, TIMP3, CTRP5, ABCA4 genes (Marmorstein et al., 2002; Klenotic et al., 2004; Mandal et al., 2006). Among these the rhodopsin P23H mutation which is linked to autosomal dominant retinitis pigmentosa (RP) has been studied in detail. This mutation leads to the formation of perinuclear aggregates, increased ubiquitination and compromise in protein degradation (Illing et al., 2002; Saliba et al., 2002). In \( E_{mut}^{+/+} \) mice also increased ubiquitination was observed suggesting a possible compromise in ubiquitin mediated protein degradation pathway. Protein trafficking defects leading to apoptosis have been implicated in other degenerative diseases. The molecular pathology underlying the photoreceptor degeneration in \( E_{mut}^{+/+} \) mice with abnormal accumulation of ELOVL4 and recruitment of ubiquitin may be similar to the pathology leading to other neurodegenerations caused by protein trafficking defects.

The studies presented here reveal a possible mechanism underlying initiation of photoreceptor death and atrophy of RPE in STGD3. However, it is not clear whether this pathology is entirely due to the abnormal protein accumulation alone. The ELOVL4 is shown to be involved in elongation of Very long chain fatty acids (VLFA) (C > 26) (Vasireddy et al., 2007, 2008) and metabolism of C32-C36 acyl phosphatidyl cholines (McMahon et al., 2007). The mutations observed in STGD3 patients exert dominant negative effect, which result in ELOVL4 aggresome formation, may lead to lack or diminished levels of functional ELOVL4. Consequently, levels of very long chain fatty acids may be depleted in the retina. The effect of depletion of VLFA in the retina is not known. The photoreceptor degeneration observed in STGD3 could be either due to the abnormal accumulation of ELOVL4 or lack of VLFA, while the RPE atrophy could be due to accumulation of lipofuscin. Additional studies are needed to understand the role of ELOVL4 in retinal pathology observed in STGD3. Studies on \( E_{mut}^{+/+} \) mice indicate that the STGD3 mutation leads to abnormal structure and function of RPE and predominant cone photoreceptor loss. The retinal pathology observed in these mice is similar to the clinical features observed in patients with STGD3. These mice will serve as a valuable model to study not only STGD3, but also other retinal degeneration diseases that are associated with abnormal accumulation of mutant proteins and/or lipofuscin.

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